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Conversion to Full Donor Chimerism Following Donor Lymphocyte Infusion Is Associated With Disease Response in Patients With Multiple Myeloma

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ABSTRACT

Donor lymphocyte infusions (DLIs) have been demonstrated to induce clinical responses in patients with relapsed multiple myeloma after allogeneic bone marrow transplantation, but the immunologic mechanisms involved have not been well characterized. In patients with chronic myelocytic leukemia (CML), remissions following DLI are invariably associated with conversion to complete donor hematopoiesis, suggesting that the target antigens of this response are expressed on both normal and CML-derived hematopoietic stem cells. In the present study, we examined hematopoietic chimerism and the complexity of the T-cell receptor (TCR) repertoire in 4 patients with relapsed multiple myeloma who received infusions of donor $CD4^+$ lymphocytes. Three of 4 patients had a clinical response that began 1 to 2 months after DLI. All 3 responding patients developed lymphocytosis at the initiation of response that was due to a 2- to 4.5-fold increase in the number of $CD3^+$ T cells. In 1 patient, this was due primarily to increases in $CD3^+$ and $CD8^+$ cells; in 2 patients, to increased numbers of $CD3^+$ and $CD8^+$ and $CD3^+$ and $CD4^+$ T cells. In all responding patients, conversion to complete donor hematopoiesis occurred in the first 2 months after DLI. The single nonresponding patient remained at 100% recipient hematopoiesis. The TCR repertoire complexity was examined by polymerase chain reaction amplification of complementary-determining region 3 (CDR3) derived from 24 Vβ gene subfamilies. In 2 patients, the initiation of myeloma response and conversion to complete donor hematopoiesis was associated with normalization of TCR complexity. Complete donor chimerism and normal TCR complexity remained stable in all patients and did not change with subsequent relapse or development of graft-versus-host disease (GVHD). Thus, conversion to full donor chimerism was temporally associated with the anti-myeloma effect of DLI but not with the development of GVHD. Nevertheless, the maintenance of stable donor hematopoiesis did not prevent disease relapse and was not associated with prolonged remission. The selective relapse of myeloma cells without concomitant return of mixed hematopoietic chimerism suggests that myeloma tumor cells in some patients develop resistance to immune destruction.

KEY WORDS

Multiple myeloma • Chimerism • Bone marrow transplantation • Donor lymphocyte infusion • T-cell repertoire

INTRODUCTION

It is now well established that infusion of donor lymphocytes provides an effective therapeutic approach in patients with relapsed hematologic malignancies after allogeneic stem cell transplantation [1-3]. Responses are noted most frequently in patients with chronic myelocytic leukemia (CML); 60% to 80% of CML patients can achieve

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complete cytogenetic and molecular remission following donor lymphocyte infusion (DLI) without other treatment [2-4]. Clinical responses are less frequent in patients with relapsed acute leukemia, but several reports have documented a relatively high rate of response in patients with multiple myeloma [5-8]. In contrast to CML, however, responses in multiple myeloma do not often result in complete remission, and myeloma relapse is frequently noted after an initial period of tumor response [9]. The different responses of various hematologic malignancies may reflect inherent differences in susceptibility to immune-mediated destruction. Alternatively, these differences may reflect the ability of malignant cells to develop resistance that circumvents an initially effective immune response. Further characterization of the target specificity of the graft-versus-leukemia (GVL) and graft-versus-myeloma (GVM) responses after DLI would help distinguish between these alternative hypotheses.

To characterize the target specificity of GVL, previous studies have examined patient-donor chimerism following DLI [10-12]. In patients with CML, clinical response and disappearance of leukemic cells has been closely associated with elimination of mixed hematopoietic chimerism and conversion to complete donor hematopoiesis [10,13,14]. This observation suggests that an immune response, directed primarily against allogeneic target antigens, possibly minor histocompatibility antigens rather than tumor-specific antigens is responsible for disease eradication after DLI [15-17]. In CML, both malignant and normal hematopoietic progenitor cells are presumed to have a similar antigenic phenotype, and this may explain the high degree of responsiveness to DLI. However, multiple myeloma tumor cells represent a lineage-restricted target and are less likely to be antigenically similar to normal hematopoietic stem cells. We therefore hypothesized that a detailed examination of hematopoietic chimerism in patients with multiple myeloma would provide additional insight into the target specificity of the GVM immune response [18].

In the present study, we examined 4 patients with relapsed multiple myeloma after allogeneic bone marrow transplantation (BMT) who received infusions of $CD4^+$ donor lymphocytes. Serial analysis of hematopoietic chimerism, phenotype of peripheral blood lymphocytes, and T-cell receptor (TCR) Vβ repertoire was undertaken in these patients. Results obtained over a 2- to 3-year period were correlated with the onset of myeloma response, graft-versus-host disease (GVHD), and relapse after DLI. These results suggest that the initial response to DLI in patients with myeloma is directed at antigens expressed by both normal hematopoietic and tumor-derived recipient cells. The frequent occurrence of relapse following DLI in multiple myeloma may be due to the development of resistance to immune-mediated destruction.

MATERIALS AND METHODS

$CD4^+$ Donor Lymphocyte Infusions

The present studies were performed in 4 patients with relapsed multiple myeloma who had undergone allogeneic BMT from an HLA-identical sibling donor. Donor marrow was depleted of $CD6^+$ T cells in vitro, and patients received no other prophylactic immunosuppressive therapy after BMT [19,20]. After relapse, each patient was enrolled in a clinical trial to evaluate the toxicity and immunologic effects of escalating doses of allogeneic $CD4^+$ lymphocytes [9]. Donor T cells were obtained by leukapheresis from the same HLA-identical sibling that had donated marrow for allogeneic BMT. The method for depletion of $CD8^+$ cells has been described [9]. Patients described in this report received either 1×10^8 or 1.5×10^8 $CD4^+$ cells/kg. DLIs contained <1% $CD8^+$ cells. DLIs were repeated at weekly intervals until the targeted number of $CD4^+$ T cells was administered. All other therapy was discontinued before DLI, and patients received no other immune-modulating therapy after DLI. The clinical research protocol for this treatment was approved by the Human Subjects Protection Committee of the Dana-Farber Cancer Institute, and informed consent was obtained from each patient and donor. Blood samples for in vitro experiments were obtained from the donors and from the patients before DLI and at different time points after DLI.

Phenotypic Analysis of Peripheral Blood Lymphocytes

Heparinized blood samples from patients were obtained before and at various times after DLI. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation, cryopreserved with 10% dimethyl sulfoxide (DMSO), and stored in vapor-phase liquid nitrogen until the phenotypic and molecular analysis. For flow cytometric analysis, 0.5 to 1×10^6 PBMCs were incubated at 4°C for 30 minutes with murine monoclonal antibodies specific for CD3, CD4, and CD8 antigens conjugated to fluorescein isothiocyanate or phycoerythrin (Coulter Immunology, Hialeah, FL). Antibodies were used at 1:100 dilution, and cells were washed with phosphate-buffered saline, followed by fixation with 2% paraformaldehyde. Immunophenotypic analysis of the stained and fixed cells was performed on a Coulter EPICS XL Flow Cytometer (Beckman Coulter, Hialeah, FL). Absolute numbers of T-cell subsets in peripheral blood samples were calculated after determining the percent positive cells by flow cytometry and the absolute number of mononuclear cells from an automated complete blood cell count differential obtained on the same day.

Hematopoietic Chimerism Assay

Genomic DNA was extracted from 3 to 10×10^6 PBMCs according to the manufacturer's recommendations (Wizard Genomic DNA Purification Kit; Promega, Madison, WI). Before amplification, DNA in each sample was quantitated by ultraviolet spectrophotometry and diluted to working concentrations. DNA from each donor-recipient pair was amplified by polymerase chain reaction (PCR) with a panel of 4 primer pairs specific for polymorphic microsatellite regions to identify an informative locus. The previously described primer sequences are designated as b7, h10, h12, h4 [21]. As a modification of the technique described by Oberkircher et al. [21], we conjugated the 3′ primer of each pair to fluorescent 5′-6 carboxyfluorescein (6-FAM) or Hex dye (Genosys Biotechnologies, The Woodlands, TX). All reactions were performed in a total volume of 50 mL, with 0.03 μg genomic DNA; 10 mmol/L Tris-HCl (pH 8.3); 1.5 mmol/L Mg_2Cl; 50 mmol/L KCl; 0.01% (wt/vol) gelatin; 200 μmol/L each of dGTP, dATP, dTTP, and dCTP; 0.5 U

AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT); and 10% DMSO. Each cycle consisted of denaturation at 94°C for 5 minutes, primer annealing at 55°C for 60 seconds, and primer extension at 72°C for 60 seconds for 40 cycles. A final 10-minute extension at 72°C followed the last cycle. The amplification products were electrophoresed on an automated 373 or 377 DNA sequencer (Applied Biosystems, Foster City, CA) using a 4.75% or 4% polyacrylamide gel, respectively, and data were analyzed by GeneScan software (Perkin Elmer Cetus Instruments, Emeryville, CA). A locus was defined as informative if analysis of recipient and donor samples before transplant showed a unique band for the recipient, a unique band for the donor, or a unique band for each. Once an informative locus was identified, genomic DNA from subsequent pre- and post-DLI samples at various time points was amplified with that specific primer to follow hematopoietic chimerism. To quantify the donor:recipient ratio in mixed chimeras, results were referred to standard amplifications of different mixtures of donor and recipient pretransplant DNA (ranging from 90:10 to 10:90).

In 1 sex-mismatched donor-recipient pair, we were not able to identify any informative microsatellite locus, and PCR amplification of the sY14-SRY (testis determining factor) gene on the Y chromosome was used to discriminate between donor (female) and recipient (male) cells. Genomic DNA was amplified in a total volume of 30 μL using the following primers: 5′-GAATATTCCCGCTCTCCGGA-3′ and 3′-GCTGGTGCTCCATTCTTGAG-5′. Thirty-five cycles of 30 seconds at 94°C, 2 minutes at 58°C, and 2 minutes at 72°C were used, with a final extension of 7 minutes at 72°C.

TCR Vβ Repertoire Analysis

PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation and cryopreserved. For TCR Vβ analysis, samples from different time points were thawed and enriched for T lymphocytes by immunomagnetic selection of $CD3^+$ cells using magnetic cell separation (MACS) beads (Milteny Biotech, Sunnyvale, CA).

Our method for TCR Vβ repertoire analysis has been described [22-24]. Briefly, RNA was extracted from T-cell preparations using the RNAStat-60 kit (Tel-Test, Friendswood, TX), and first-strand cDNA was generated from 2 μg total RNA using random hexanucleotides (Pharmacia Biotech, Piscataway, NJ) and reverse transcriptase (Superscript, Gaithersburg, MD). Each Vβ segment was amplified with 1 of the 26 Vβ subfamily–specific primers and a Cβ primer recognizing both Cβ1 and Cβ2 regions. The Cβ primer was conjugated to fluorescent dye 6-FAM (Applied Biosystems, Foster City, CA) for complementarity-determining region 3 (CDR3) size analysis. The amplification was performed over 30 cycles in a final volume of 100 μL on a DNA thermal cycler (Perkin-Elmer, Norwalk, CT).

TCR β Chain CDR3 Fragment Size Analysis

The size distribution of each fluorescent PCR product was determined by electrophoresis on an automated 373 DNA sequencer (Applied Biosystems) using a 5.5% polyacrylamide gel and the data analyzed by GeneScan software (Perkin Elmer, Foster City, CA). Because the position of the 5′ and 3′ primers are fixed, fragment size differences within each Vβ subfamily are due entirely to different complementary-determining region 3 (CDR3) region lengths, reflecting junctional diversity and N-random nucleotide insertions in the V-D-J region. Peaks corresponding to in-frame transcripts are detected at 3-nucleotide intervals. As described previously, a normal transcript size distribution, reflecting polyclonal cDNA, contains 8 to 10 peaks for each TCR Vβ subfamily with a Gaussian size distribution [22-26]. The appearance of dominant peaks indicates the presence of excess cDNA of identical size, suggesting the presence of an oligoclonal or clonal T-cell population. The absence of any peaks after PCR amplification indicates the absence of any T cells using a specific Vβ subfamily of TCR genes.

TCR β Chain CDR3 Complexity Scoring

The overall complexity within a TCR Vβ subfamily was determined by counting the number of discrete peaks per subfamily [24]. Subfamilies were graded on a score of 0 to 8 based on the degree of complexity. A score of 0 was assigned if a subfamily was absent, a score of 1 was given if a subfamily demonstrated only a single monoclonal peak, a score of 2 was given for a biclonal profile, and so on. Finally, a score of 8 denoted a "normal" appearing CDR3 pattern of 8 to 10 peaks with a complex, diverse, and polyclonal appearance. The overall TCR Vβ complexity score per sample was calculated as the summation of the number of subfamilies per score category, with a maximum possible score of 208 (8×26). Based on the TCR Vβ complexity scores of 10 normal donors, we established a score of <142 as abnormal, based on the 95% lower confidence interval.

RESULTS

Clinical Myeloma Response After $CD4^+$ DLIs

In the present study, we examined 4 patients with relapsed multiple myeloma who were enrolled in a clinical trial to determine the toxicity and immunologic effects of infusion of defined doses of $CD4^+$ donor T cells. In this trial, it was noted that hematologic responses occurred primarily in patients with CML and multiple myeloma [9]. To examine the immunologic effects of DLI associated with clinical response, we selected for further analysis 3 patients with multiple myeloma who demonstrated either a complete or partial response following DLI and 1 patient without evident tumor response. Clinical characteristics of these 4 patients and their course after BMT are summarized in the Table. Myeloma response after DLI was evaluated by serial measurement of immunoglobulin (Ig) levels and monoclonal paraprotein in serum and by serial histologic examination of bone marrow aspirates and biopsies. Serum immunoglobulin levels in patients 1, 2, and 4 after DLI are summarized in Figure 1. Patient 3 had light chain disease without detectable serum paraprotein. Myeloma response in patient 3 was evaluated by histologic examination of serial marrow biopsies and examination of urine for the presence of monoclonal light chain.

At 1 month after DLI, patient 1 (Figure 1A) was found to have decreasing levels of serum paraprotein. Total serum IgG and myeloma paraprotein levels continued to decrease slowly over the next year, and marrow biopsies demonstrated a histologic remission 10 months after DLI. However, paraprotein remained detectable in serum, and a complete

*Patient Characteristics**

Patient	Age, y	Sex	Donor Sex	Myeloma Paraprotein	Treatment Before BMT	Status at BMT	BMT to Relapse, mo	Relapse to DLI, mo	CD4 Cells Infused, 10^8/kg
1	40	M	F	IgGλ	MP Dex HD-Cy	Minimal disease	6	3	1
2	42	M	F	IgAκ	VAD	Minimal disease	53	9	1.5
3	46	F	F	Light chain λ	VAD MP HD-Cy	Partial response	38	5	1
4	56	M	M	IgGλ	MP CBVM VAD HD-Cy	Minimal disease	9	7	1.5

*BMT indicates bone marrow transplant; DLI, donor lymphocyte infusion; Ig, immunoglobin; MP, melphalan, prednisone; Dex, dexamethasone; HD-Cy, high-dose cyclophosphamide; VAD, vincristine, doxorubicin, dexamethasone; CBVM, cyclophosphamide, carmustine, vincristine, melphalan.

response was not demonstrated until 2 years after DLI. Beginning 5 months after DLI, this patient developed lichenoid GVHD of the skin and chronic GVHD of the liver, requiring treatment with prednisone and cyclosporine. This patient continues to have evidence of chronic skin GVHD and also continues in complete remission without evidence of increased plasma cells in marrow and without detectable serum paraprotein 4 years after DLI.

Patient 2 (Figure 1B) continued to have increasing levels of serum monoclonal IgA paraprotein 1 month after DLI, but decreasing levels were noted 2 months after DLI. The patient quickly reached normal serum levels of IgA, but monoclonal protein was always detectable and he never attained a complete remission. Marrow biopsies during this period demonstrated 5% to 10% plasma cells that expressed a monoclonal light chain. Four months after DLI, he developed extensive GVHD of the skin and liver, requiring treatment with prednisone and cyclosporine. The patient remained in a minimal disease state for 1.5 years after DLI, when progressive disease was again documented. The initial site of disease progression was a pleural plasmacytoma.

Patient 3 did not have detectable monoclonal paraprotein in serum, but disease regression became evident with decreased urinary excretion of light chain 2 months after DLI. A complete response was noted 6 months after DLI, with absence of urinary light chain excretion and histologically normal marrow biopsy. The patient relapsed with a single lytic bone lesion and sinus cavity plasmacytoma 8 months after DLI. This was treated with local radiation therapy. Patient 3 developed extensive chronic GVHD 10 months after DLI that continued despite treatment with prednisone and cyclosporine.

Patient 4 (Figure 1C) developed a bone plasmacytoma 1 month after DLI. This was treated with dexamethasone and local radiation therapy. Serum paraprotein levels continued to increase for 2 months after DLI but decreased after this additional systemic and local treatment. He continued to have stable disease until increasing serum paraprotein levels developed 6 months after DLI. No response was noted after a second DLI. This patient did not develop GVHD after either the first or second DLI.

Expansion of Peripheral Blood T Cells After DLI

Changes in peripheral blood lymphocytes were monitored by serial assessment of the phenotype of circulating T cells after DLI. Patient 1 was found to have increased numbers of circulating lymphocytes 1 month after DLI, and patients 2 and 3 developed lymphocytosis 2 months after DLI. In these 3 cases, peripheral lymphocytosis coincided with the initiation of a tumor response, with no lymphocytosis noted in patient 4. The results of the phenotypic analysis of PBMCs in the first 3 months after DLI for all 4 patients is summarized in Figure 2. When compared with baseline levels before DLI, patient 1 was found to have a 2-fold increase in $CD3^+$ cells 1 month after DLI. These cells were predominantly $CD8^+$. Patients 2 and 3 were noted to have 2- and 4.5-fold increases, respectively, in $CD3^+$ cells 2 months after DLI. In both of these patients, this change was due to increases in both $CD4^+$ and $CD8^+$ cells. Similar changes in circulating T cells were not noted in patient 4.

Changes in Hematopoietic Chimerism After DLI

The relative contributions of recipient and donor hematopoiesis were examined by serial analysis of polymorphic microsatellite loci by PCR amplification of genomic DNA obtained from patient PBMCs. In patient 2, no informative patient-donor microsatellite pair was identified, and we therefore used a Y chromosome-specific nonquantitative PCR assay to distinguish between patient (male) and donor (female) cells. As shown in Figure 3A, patient and donor DNA could be easily distinguished by the presence of unique profiles using this method in patient 1. Before DLI, no evidence of donor DNA could be detected indicating that PBMCs were entirely derived from recipient hematopoiesis. In contrast, PBMCs obtained 1 month after DLI contained only donor DNA, indicating rapid conversion to complete donor hematopoiesis in this patient. Results of PCR amplification of a Y chromosome-specific marker in patient 2 also indicated the presence of recipient cells before and at 1 month after DLI (Figure 3B). Although this sensitive assay does not provide an assessment of the relative contribution of recipient cells at these 2 time points, all subsequent PCR assays beginning at 2 months after DLI no

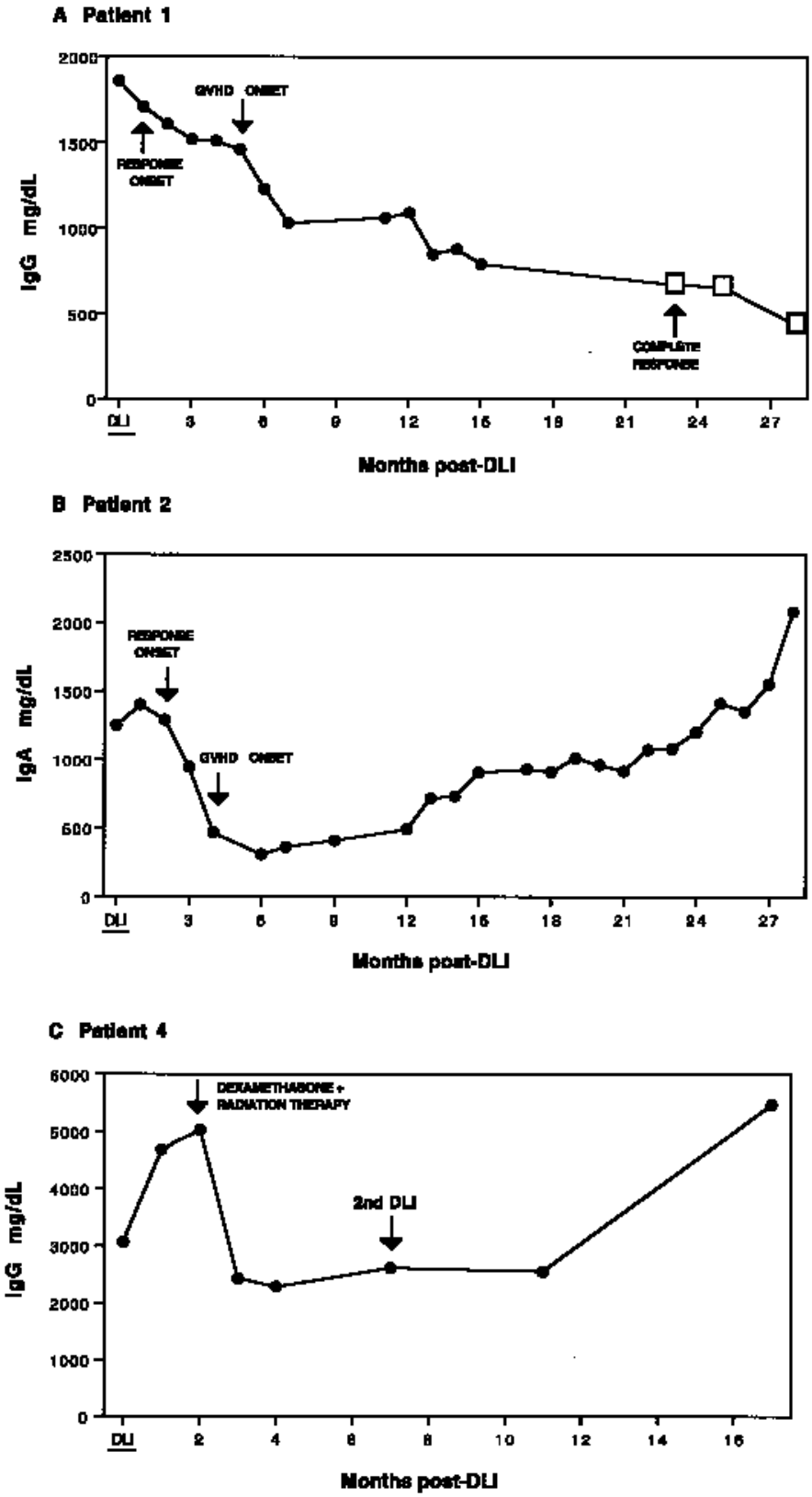

Figure 1. Measurement of disease status after donor lymphocyte infusion (DLI). A. Serum levels of immunoglobulin G (IgG) for patient 1. B. Serum levels of IgA for patient 2. C. Serum levels of IgG for patient 4. Serum levels are reported at the time of DLI and in the following months. □ indicates a complete remission (no detection of monoclonal protein); ●, the presence of monoclonal paraprotein. The onset of graft-versus-host disease (GVHD), clinical response, and additional therapies are also indicated.

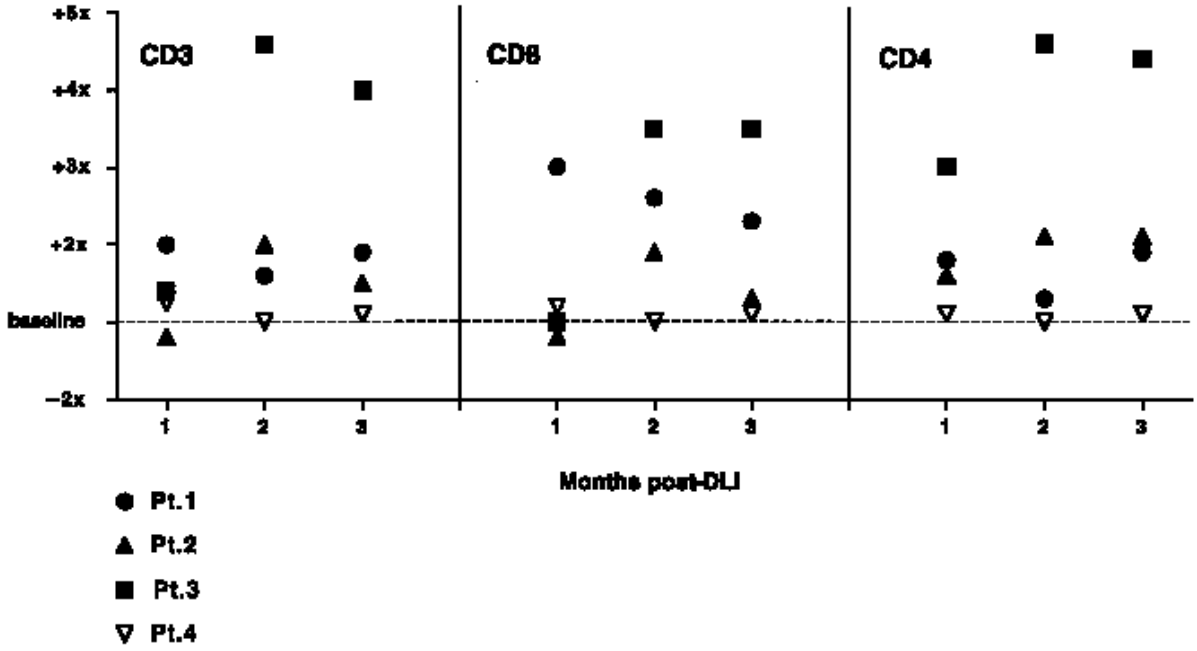

Figure 2. Changes in circulating T cells after donor lymphocyte infusion (DLI). Absolute numbers of $CD3^+$, $CD8^+$, and $CD4^+$ cells were calculated after determining the absolute number of mononuclear cells and percent positive cells by immunostaining. The relative increase or decrease of each population in comparison to baseline values obtained before DLI is represented for every patient (Pt.).

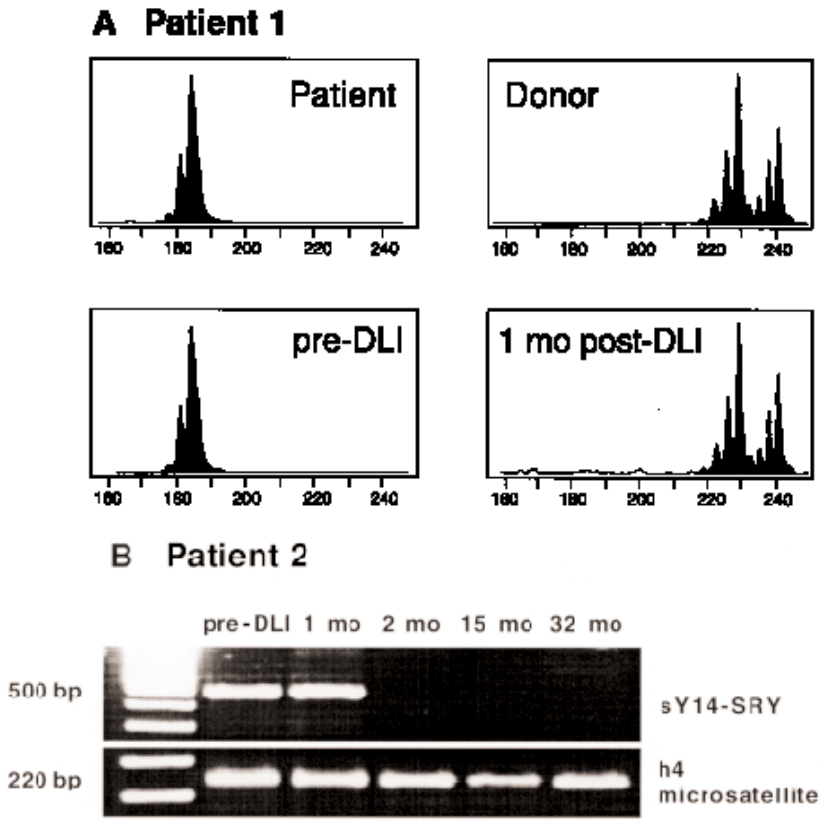

Figure 3. Hematopoietic chimerism after donor lymphocyte infusion (DLI). A. Detection of donor and patient-derived microsatellite polymorphisms in serial blood samples from patient 1. Results from patient and donor blood samples before bone marrow transplantation, before DLI, and 1 month after DLI. B. Detection of Y chromosome–specific DNA (sY14-SRY) indicating the presence of recipient cells in serial peripheral blood samples from patient 2. bp indicates base pair.

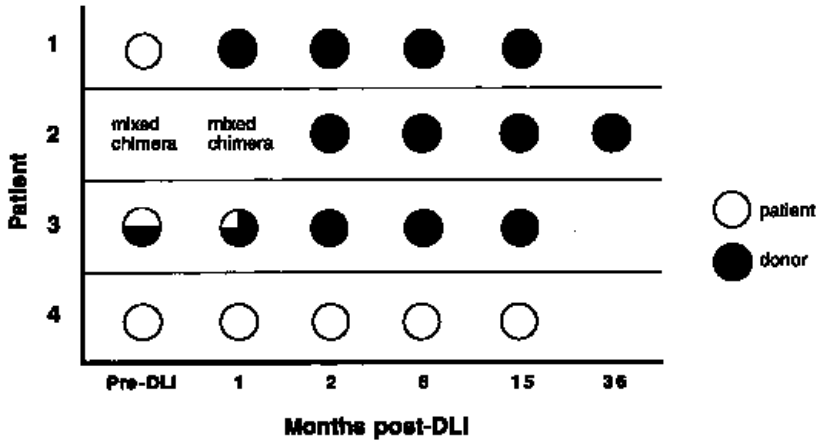

Figure 4. Summary of changes in hematopoietic chimerism after donor lymphocyte infusion (DLI). The relative contribution of patient and donor hematopoiesis in serial blood samples pre- and post-DLI is represented for every patient. In patient 2, the nonquantitative nature of the polymerase chain reaction method used does not allow the precise quantitation of the patient-derived component detected pre-DLI.

longer detected the presence of residual recipient cells in PBMCs. This indicated a conversion to complete donor hematopoiesis at 2 months after DLI and the subsequent maintenance of this status.

The results of all chimerism assays for the 4 patients are summarized in Figure 4. All 4 myeloma patients had evidence of substantial mixed chimerism in PBMCs before DLI. In patients 1 and 4, donor cells could not be detected using this sensitive method. Patients 1, 2, and 3 rapidly converted to complete donor hematopoiesis within 2 months after DLI. In contrast, patient 4 showed persistence of complete recipient hematopoiesis throughout the 15-month period of observation.

TCR Repertoire Complexity After DLI

The complexity of the T-cell repertoire after DLI was examined by analysis of PCR-amplified products of each of the 24 TCR Vβ subfamilies. Two Vβ subfamilies (Vβ5 and Vβ13) require 2 sets of variable region primers to visualize the entire subfamily gene, thus producing 26 different profiles for each sample analyzed. This method specifically amplifies the CDR3 region used by each mature T cell. Normal polyclonal populations, therefore, exhibit a diverse pattern of 8 to 10 peaks in a Gaussian distribution for each Vβ subfamily [25,26]. The results of this analysis for peripheral blood samples obtained before DLI and 1 month after DLI in patient 1 are shown in Figure 5. Although some Vβ families exhibited normal profiles (Vβ4, Vβ5, Vβ9), the majority of profiles in the pre-DLI sample were abnormal, exhibiting clonal (Vβ14), oligoclonal (Vβ16), or absent (Vβ23) profiles indicative of a markedly restricted T-cell repertoire. In contrast, the 1-month post-DLI sample demonstrated a normal TCR repertoire in almost all profiles. Although some clonal (Vβ 24) and oligoclonal (Vβ 16) profiles persisted, the sample overall indicated a relatively rapid improvement in the complexity of the TCR repertoire in this interval.

To compare changes in TCR repertoire complexity in different patients and in different samples after DLI, we used a scoring system to quantify the degree of complexity of each CDR3 profile [24]. In previous studies, analysis of samples from 10 normal donors was used to define a score of 142 as the lower limit of normal. Results of this analysis for peripheral blood lymphocyte (PBL) obtained from each of the 4 myeloma patients for 1 year after DLI are shown in Figure 6. Patients 2 and 4 had relatively normal complexity scores before DLI and did not demonstrate any substantial changes in repertoire complexity after DLI. In contrast, patients 1 and 3 had significantly low complexity scores before DLI. Repertoire complexity normalized at 1 month after DLI in patient 1 and at 2 months after DLI in patient 3. This normalization of TCR repertoire was maintained for at least 1 year in both patients.

Correlation of Hematopoietic Chimerism With Myeloma Response and GVHD

The results of our analysis of hematopoietic chimerism are summarized in Figure 7 and shown in relation to the demonstration of myeloma response and the development of GVHD in each patient. Patient 4 continued to demonstrate predominantly, recipient hematopoiesis throughout this period. No changes in repertoire complexity were observed, and neither a myeloma response nor GVHD developed after DLI. In contrast, patients 1, 2, and 3 demonstrated conversion to complete donor hematopoiesis 1 to 2 months after DLI. This conversion coincided with the initiation of clinical myeloma response. In patients 1 and 3, the conversion to donor hematopoiesis also coincided with the normalization of TCR repertoire complexity. Changes in hematopoietic chimerism and TCR repertoire did not appear to coincide with the onset of either acute or chronic GVHD after DLI. With subsequent relapse of myeloma cells in patients 2 and 3, there was no change in hematopoietic chimerism, and these patients continued to demonstrate persistence of complete donor hematopoiesis.

DISCUSSION

The clinical responses to DLI in patients with relapsed multiple myeloma provide direct evidence for the suscepti-

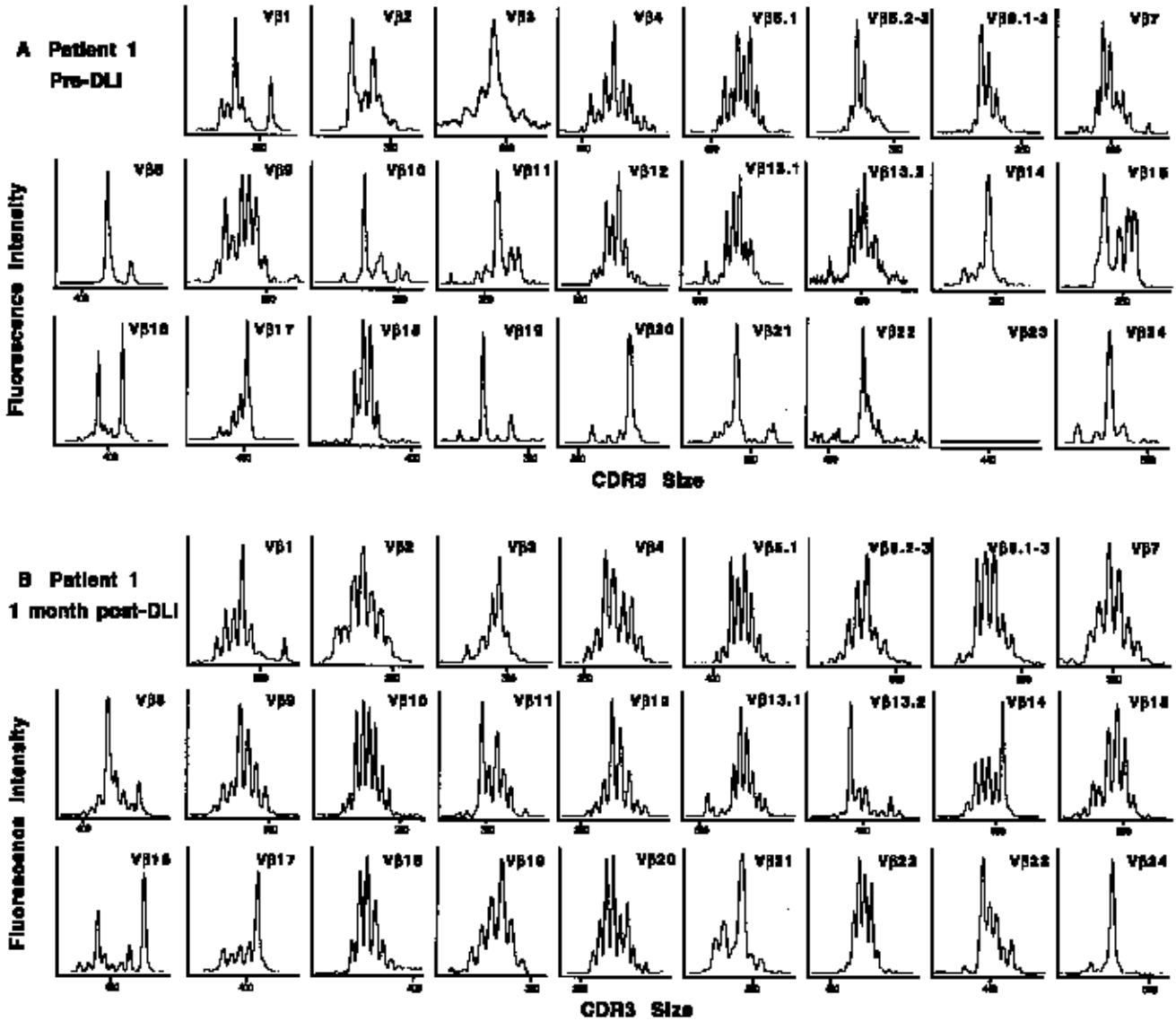

Figure 5. T-cell receptor (TCR) complementarity-determining region 3 (CDR3) diversity in peripheral blood T cells. A. CDR3 size distribution profiles for each of 24 TCR Vb gene subfamilies for samples obtained before donor lymphocyte infusion (DLI). B. Comparison with results obtained 1 month post-DLI in patient 1.

bility of multiple myeloma cells to immunotherapeutic approaches using adoptive transfer of allogeneic T cells [5,8,9]. Nevertheless, the immunologic mechanisms that mediate this GVM effect, as well as the target antigens for this response, have not been established. The present studies were undertaken to characterize the immunologic effects of donor T-cell infusions in patients with multiple myeloma. These studies demonstrated that an effective immune response directed against myeloma tumor cells began approximately 4 to 8 weeks after DLI and coincided with the elimination of normal recipient hematopoietic cells and conversion to complete donor hematopoiesis. The association of clinical response with the establishment of complete donor hematopoiesis has previously been observed in patients with CML responding to DLI [10,11,13]. Although it is possible that the immune responses against tumor cells and normal hematopoietic progenitors are distinct, the present results suggest that the antitumor response in patients with multiple myeloma is directed against target antigens expressed on normal hematopoietic progenitor cells as well as malignant cells in the recipient. Interestingly, examination of hematopoietic chimerism in the single patient who did not respond to DLI demonstrated no change in chimerism and persistence of predominant recipient hematopoiesis. Although we were able to examine only a single nonresponding patient, this finding suggests that the lack of antimyeloma response in this individual may be due to the inability to generate an immune response directed against common antigens expressed on recipient hematopoietic cells rather than resistance of the myeloma cells to immune-mediated destruction. Alternatively, it is also possible that the lack of immunologic effect may have been due to rejection of donor cells. No phenotypic changes associated with rejection were observed after DLI [27], but studies

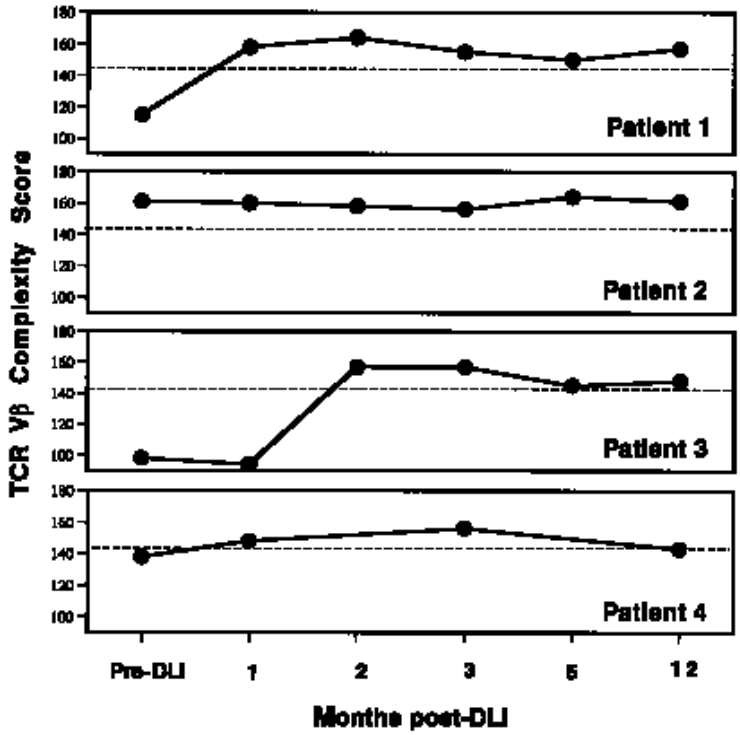

Figure 6. Summary of T-cell receptor (TCR) repertoire complexity scores after donor lymphocyte infusion (DLI). The overall TCR complexity score for all 24 Vb gene subfamilies was calculated for peripheral blood samples obtained in every patient before DLI and at various times after DLI. The dotted line represents the lower limit of the normal range of TCR Vb complexity scores for 10 normal individuals.

to examine this alternative explanation for resistance to DLI should be carried out to address this issue.

Further analysis in these selected individuals also demonstrated that the 3 responding patients developed varying degrees of T-cell lymphocytosis at the time that the antimyeloma response became clinically apparent. Although the DLI consisted of defined numbers of $CD4^+$ T cells that had been depleted of $CD8^+$ cells ex vivo, the lymphocytosis consisted primarily of $CD8^+$ cells in 1 patient and of both $CD4^+$ and $CD8^+$ T cells in the other 2 responders. Interestingly, this lymphocytosis was noted 4 to 8 weeks after DLI and also occurred as the patients were converting from mixed chimerism to complete donor hematopoiesis. These cells in peripheral blood were therefore of donor origin, but it is not known whether they were directly derived from the lymphocyte infusion or whether they may have been derived from cells that had previously engrafted during the primary allogeneic marrow transplant. The finding that many of these cells were $CD8^+$ suggests that they were not contained in the DLI, which had been depleted of $CD8^+$ cells before infusion. However, because these cells are all derived from the same normal donor, these 2 alternative possibilities can only be distinguished in experiments in which genetic markers are introduced into the DLI product before infusion [28,29]. Such experiments would be very helpful in determining whether the $CD4^+$ DLI produced a direct antitumor effect or whether these cells primarily acted by providing T-cell help, with subsequent expansion of an effector population that had previously been suppressed or otherwise unable to mediate an effective antimyeloma response in vivo.

Another striking finding that coincided with the initiation of the antimyeloma response after DLI, was normalization of the TCR repertoire in 2 of the 3 responding patients. In previous studies in patients with CML and multiple myeloma, we found that serial analysis of TCR Vβ repertoire revealed the expansion of clonal T-cell populations in peripheral blood after DLI [22,23]. These clonal T-cell populations became evident at the initiation of the antitumor response and persisted for prolonged periods. Distinct clonal T-cell populations could be detected in peripheral blood when clinical GVHD developed. Normalization of TCR repertoire was also noted in CML patients after DLI, but improvement in TCR repertoire occurred very slowly in responding individuals: approximately 1 year was required before a normal TCR repertoire was achieved [22]. A similar time period is required for normalization of TCR repertoire after primary allogeneic stem cell transplantation [24]. The rapidity of the improvement in TCR repertoire in the patients with multiple myeloma may indicate that the polyclonal T cells appearing in the peripheral blood after DLI, in these patients, do not represent the differentiation of new T cells from hematopoietic progenitor cells. Abnormal TCR profiles, such as those seen in patients 1 and 3, may be due to the presence of oligoclonal T-cell expansions that obscure the presence of polyclonal T cells in the assay. In these 2 patients, normalization of TCR repertoire coincided with the conversion to complete donor hematopoiesis. A recent analysis of TCR repertoire after allogeneic BMT demonstrated that abnormal TCR repertoire profile may be due to the relative overexpansion of

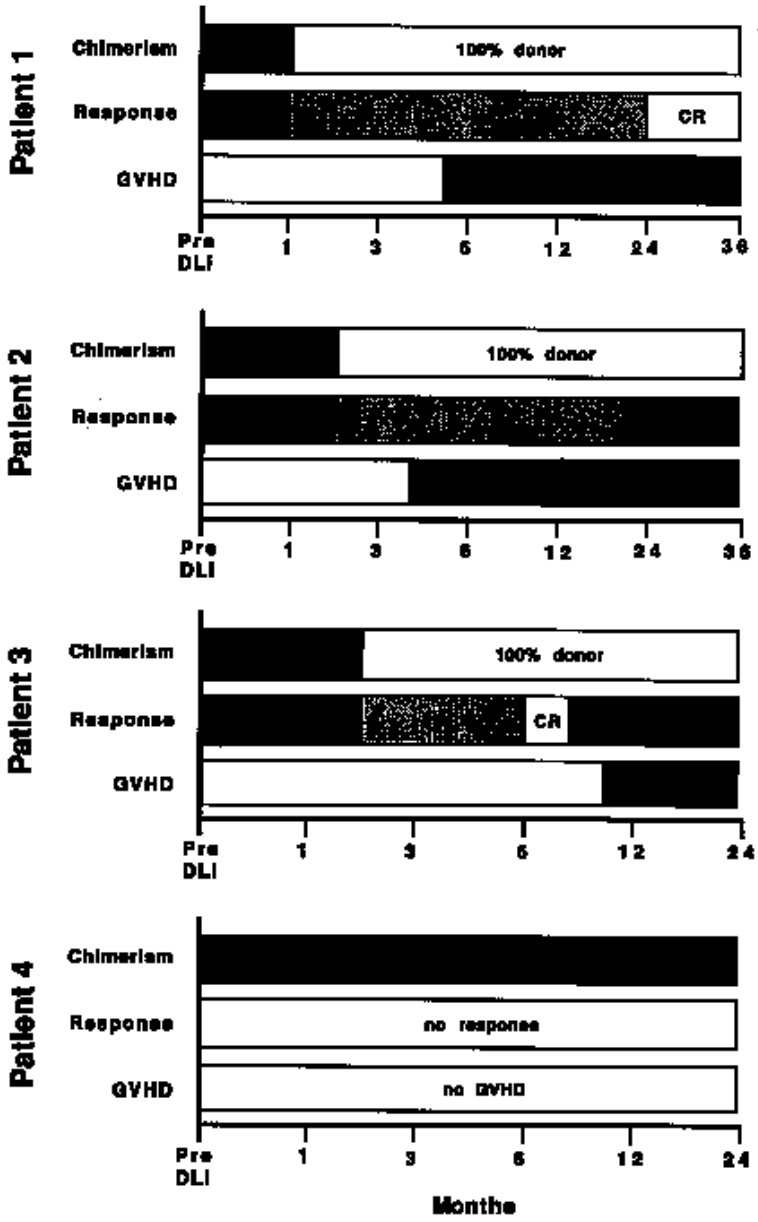

Figure 7. Correlation of changes in hematopoietic chimerism with myeloma response and graft-versus-host disease (GVHD) after donor lymphocyte infusion. CR indicates complete response; PR, partial response; pt, patient.

oligoclonal T cells, rather than the loss of a polyclonal repertoire [30]. Thus, the elimination of recipient-derived oligoclonal T cells in our patients after DLI appeared to allow the rapid expansion of polyclonal donor-derived T cells and reestablishment of a normal T-cell repertoire. This is a more likely explanation for the normalization of TCR repertoire in our patients than the generation of new T cells from undifferentiated lymphocyte progenitor cells.

T cells are presumed to be the principal mediators of GVL activity as well as GVHD, but it is not known whether GVL and GVHD are identical or distinct immunologic effects. To address this issue, 2 clinical trials examined whether depletion of $CD8^+$ T cells from the DLI can selectively reduce the incidence of GVHD without affecting GVL activity [9,31]. Interestingly, both of these studies suggested that selective infusion of $CD4^+$ donor T cells reduces the risk of acute and chronic GVHD without eliminating GVL activity. Nevertheless, 3 of the 4 patients described in this report developed GVHD after CD8-depleted DLI. Each of these patients, however, received relatively large numbers of donor cells, and GVHD occurred several weeks or months after the initial response against myeloma cells began. Thus, both the antimyeloma response and the conversion to complete donor hematopoiesis occurred before any clinical evidence of GVHD was observed. Moreover, the persistence of active GVHD did not prevent myeloma relapse in 2 patients. Taken together, these observations provide support for the notions that GVHD is a distinct

immunologic process and that it may be possible to develop methods for selectively enhancing GVL without concomitant increase in the toxicity associated with GVHD. Further characterization of both of these immunologic responses is necessary to determine the immunologic basis for these clinical observations.

Although the antimyeloma response became evident relatively soon after DLI, not all responding patients achieved a complete response, and prolonged periods of 1 to 2 years were required before this could be achieved. The prolonged period required to eradicate relatively small numbers of residual myeloma cells is similar to the time required to achieve molecular remission in patients with CML. However, this appears to be very different from the response directed against recipient hematopoietic cells, which resulted in the very rapid and complete elimination of relatively large numbers of normal cells. Although the method for quantitation of hematopoietic chimerism in patients 1, 3, and 4 was not developed to detect small numbers of recipient cells, the PCR assay used in patient 2 targeted a Y chromosome–specific antigen and therefore provides a very sensitive method for detecting residual recipient cells. This assay does not allow us to provide an accurate estimate of the percentage of recipient cells in the samples tested, but once conversion to donor hematopoiesis occurred, no residual recipient male cells could be detected in repeated tests. Thus, in contrast to the relatively slow elimination of myeloma cells, these observations indicate that complete elimination of residual recipient hematopoiesis occurred very rapidly after DLI.

Unfortunately, 2 of the patients who converted to complete donor hematopoiesis subsequently relapsed. Relapse occurred initially in extramedullary sites, but despite reemergence of myeloma tumor cells, blood samples in both individuals remained entirely of donor origin. Although the reasons for relapse in these individuals have not been identified, this observation suggests that relapse of myeloma may be due to the development of resistance to immune destruction rather than to suppression of the donor immune response. Similar observations in patients with relapsed melanoma after immunotherapy have previously identified several mechanisms whereby tumor cells can become resistant to immune-mediated attack [32,33]. Further studies will be necessary to identify the target antigens of this response as well as the mechanisms of tumor escape. With further characterization of the antimyeloma response in additional patients, it may be possible to improve the durability of the antitumor response and extend this immunotherapeutic approach to other patient populations that do not currently respond to this treatment.

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